Designation: E3143 – 18b (Reapproved 2023)

Standard Practice for Performing Cryo-Transmission Electron Microscopy of Liposomes[1]

This standard is issued under the fixed designation E3143; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This practice covers procedures for vitrifying and recording images of a suspension of liposomes with a cryo-transmission electron microscope (cryo-TEM) for the purpose of evaluating their shape, size distribution and lamellarity for quality assessment. The sample is vitrified in liquid ethane onto specially prepared holey, ultra-thin, or continuous carbon TEM grids, and imaged in a cryo-holder placed in a cryo-TEM.

1.2 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.4 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E456 Terminology Relating to Quality and Statistics

2.2 *ISO Standards:*[3]

13322-1 Particle Size Analysis – Image Analysis Methods – Static Image Analysis Methods

3. Terminology

3.1 *Definitions:*

3.1.1 *anti-contaminator, n*—a specially designed device built into the column of a cryo-transmission electron microscope designed to pull contamination produced by outgassing within the column away from the frozen specimen during imaging. The device is cooled with liquid nitrogen to a temperature below that of the specimen creating a colder surface for contamination to build on.

3.1.2 *carbon evaporator, n*—a device used to evaporate carbon in a high vacuum chamber generally by applying current through two carbon rods pressed against one another, with one rod being sharpened in order to provide resistance to the current; this causes the rods to heat up and evaporate carbon. The same device can also be used as a *glow discharge device* (3.1.15) in order to *glow discharge* surfaces.

3.1.3 *continuous carbon grid, n*—a copper electron microscope grid coated with a self-supporting layer of continuous carbon over the square mesh of the grid.

3.1.4 *copper electron microscope grid, n—commonly referred to as an "EM grid,"* a thin 3 mm diameter copper foil disk, usually manufactured with a pattern of square holes called a 'mesh' through which imaging is conducted in an electron microscope. The number, pattern, and shape of the holes can vary depending on imaging conditions and sample requirements.

3.1.5 *cryo-grid storage device, n*—any device used to store cryo-EM grids indefinitely in a liquid nitrogen dewar.

3.1.6 *cryo-TEM, n—also referred to simply as cryo-EM, or cryo electron microscopy*, the process of imaging frozen hydrated, vitrified nanomaterial using a cryo-transmission electron microscope.

3.1.7 *cryo-TEM holder, n*—a liquid nitrogen refrigerated device specifically designed to hold and maintain a prepared grid containing a frozen, hydrated, vitrified specimen in a cryo-TEM while imaging is conducted.

3.1.8 *cryo-TEM plunger, n*—a device used to vitrify a sample onto a holey, ultra-thin, or continuous carbon grid for cryo-TEM by plunging the grid containing a thin layer of aqueous sample into an ethane slush or other cryogen(s). There

[1] This practice is under the jurisdiction of ASTM Committee E56 on Nanotechnology and is the direct responsibility of Subcommittee E56.02 on Physical and Chemical Characterization.

Current edition approved Sept. 1, 2023. Published September 2023. Originally approved in 2018. Last previous edition approved in 2018 as E3143 – 18b. DOI: 10.1520/E1343-18BR23.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from International Organization for Standardization (ISO), ISO Central Secretariat, BIBC II, Chemin de Blandonnet 8, CP 401, 1214 Vernier, Geneva, Switzerland, http://www.iso.org.

are many types of plunger designs both homemade and commercial. The simplest is the homemade guillotine type (see Fig. 3(*B*), 8.3).

3.1.9 *cryo-transmission electron microscope, n*—a specially designed *transmission electron microscope* (see 3.1.25) capable of imaging frozen vitrified specimens under low electron dose conditions in order to prevent beam-induced specimen damage.

3.1.10 *electron micrograph, n*—the individual image recorded by the electron microscope as data are collected.

3.1.11 *electron tomography, n*—the process of using images derived from an electron microscope in which the images were recorded of a sample through an incremental series of tilt angles that are then used with image analysis software to reconstruct the structure of the specimen in 3D.

3.1.12 *ethane, n*—chemical formula C_2H_6. A colorless, odorless flammable gas.

3.1.13 *forceps, n*—a very fine pointed pair of tweezers or pincer used to pick up electron microscope grids.

3.1.14 *glow discharge, n*—a plasma generated by passing an electric current through a low-pressure gas environment within a chamber, usually a bell jar and with respect to this practice, is used to clean and statically charge the carbon surface coating of copper electron microscope grids in order to make them hydrophilic so that they will wet during sample application.

3.1.15 *glow discharge device, n*—a device, such as a *carbon evaporator* (see 3.1.2), designed to generate a glow discharge plasma.

3.1.16 *hang time, v*—the amount of time after blotting of the sample from the prepared grid, prior to plunging the grid into the liquid ethane for vitrification.

3.1.17 *holey carbon grid, n*—a copper electron microscope grid used for cryo-TEM consisting of a thin electron semi-transparent carbon film containing small holes that is suspended over the larger mesh of square holes of the copper electron microscope grid (see Fig. 3(*A*), 8.2.1).

3.1.18 *image analysis, n*—the process of analyzing digital images with computer software for the purpose of extracting meaningful information from the data, for example, a size distribution.

3.1.19 *liposomes, n*—microvesicles composed of a bilayer and/or a concentric series of multiple bilayers separated by aqueous compartments formed by amphipathic molecules such as phospholipids that enclose a central aqueous compartment.

Liposome Drug Products (1)[4]

3.1.20 *liquid ethane, n*—liquefied ethane made by cooling gaseous ethane to the liquid state.

3.1.21 *liquid nitrogen, n*—a cryogen, is the liquid state of nitrogen that boils at –196 °C and is commonly used for extreme cooling. Poses a freezing and suffocation hazard requiring caution when used.

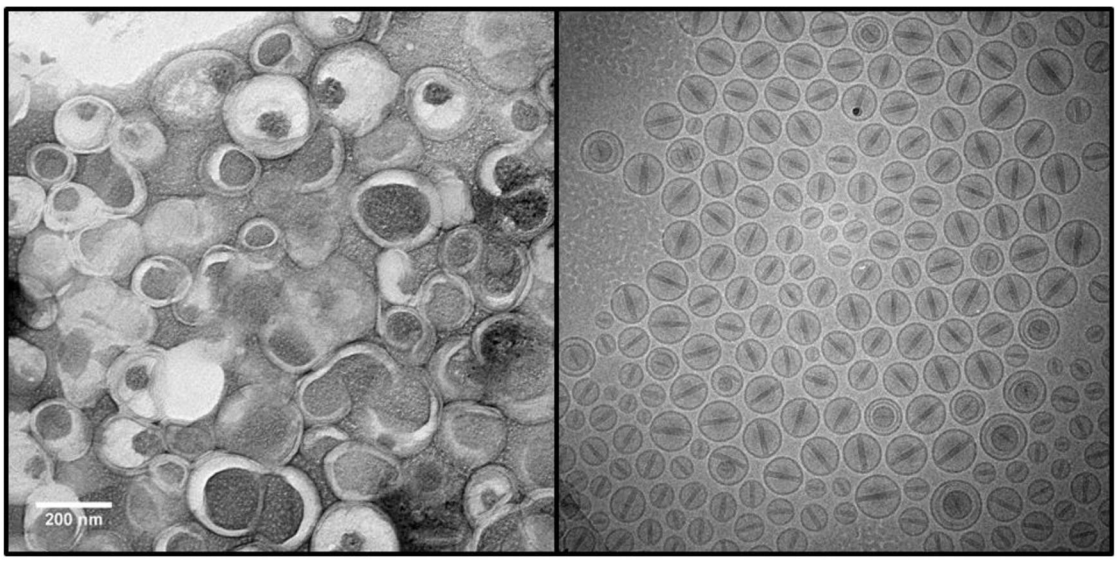
3.1.22 *sample, n*—a small volume of a preparation of liposomes suspended in an aqueous solution.

3.1.23 *specimen, n*—a cryo-TEM grid onto which a sample has been applied and vitrified. The specimen will be placed in a cryo-TEM holder and imaged by cryo-TEM.

3.1.24 *structure, n*—the 3D shape, arrangement, composition, and construction of any element that has physical *x*, *y*, and *z* dimensions.

3.1.25 *transmission electron microscope, n*—a microscope that employs an electron beam and a series of electro-magnetic lenses to illuminate [transmit] through very thin samples and then image these samples to extremely high resolutions and high magnifications.

[4] The boldface numbers in parentheses refer to a list of references at the end of this standard.



NOTE 1—Both images are shown to the same scale; scale bar is 200 nm.

FIG. 1 Left—An Electron Micrograph of an Air-Dried Liposomal Preparation that has been Negatively Stained with 2 % Uranyl Acetate for Contrast; Right—An Electron Micrograph of the Same Liposomal Preparation Prepared as a Frozen Vitrified Specimen for Cryo-TEM

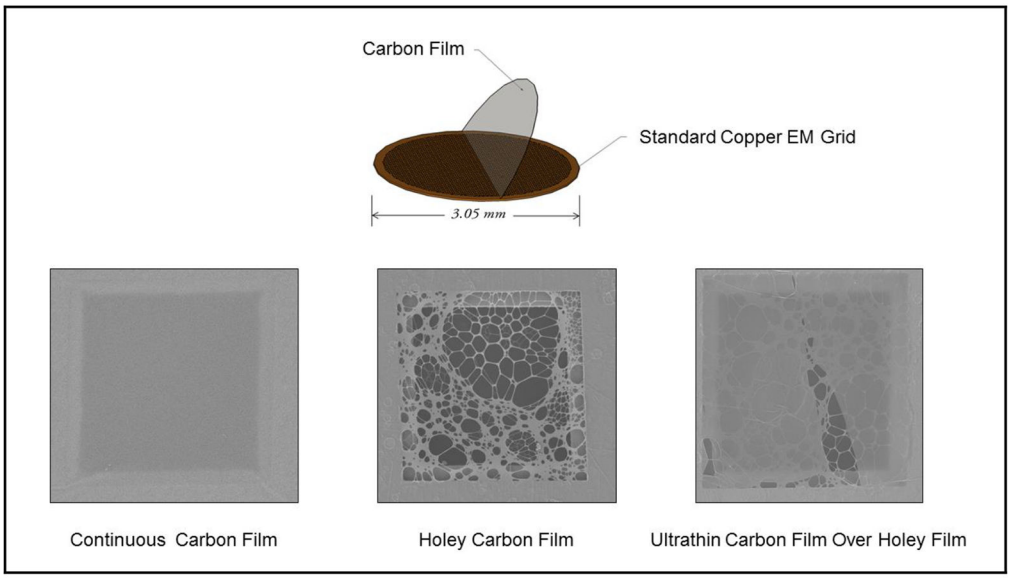


FIG. 2 The Three Different Types of Carbon Film on EM Grids That May be Used to Conduct Cryo-TEM of Liposomes

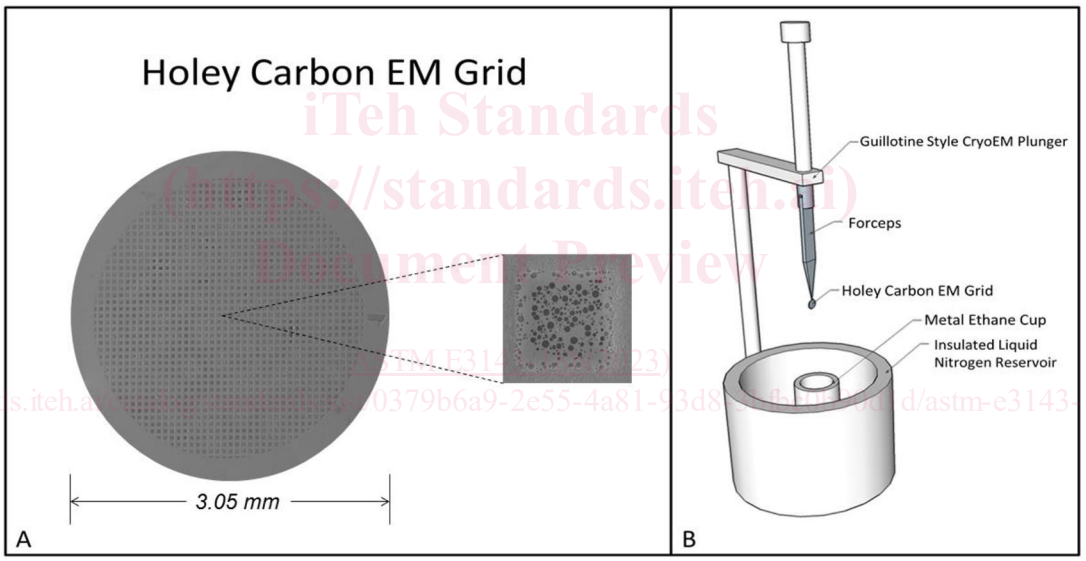


FIG. 3 (*A*) A Holey Carbon EM Grid with an Expanded View of Holey Carbon Film Shown to the Right; (*B*) Guillotine-Style Cryo-TEM Plunger Showing the Forceps Aligned with the Ethane Cup, and the Liquid Nitrogen Reservoir

3.1.26 *ultra-thin carbon grid, n*—a holey carbon grid additionally coated with a very thin and fragile layer of carbon that is supported by the thicker more sturdy holey carbon film.

3.1.27 *vitrification, n*—a process of vitrifying, that is, freezing a sample so rapidly that ice crystals do not have sufficient time to form around the sample. The resulting sample is said to be vitrified, essentially embedded in amorphous glass like ice.

4. Summary of Practice

4.1 In this practice, cryo-TEM imaging of liposomes is conducted by applying a small volume (sample) of a preparation of liposomes suspended in an aqueous solution to a freshly glow discharged TEM grid (for example, holey, or continuous carbon TEM grid, or a freshly prepared ultra-thin carbon EM grid), then vitrifying the sample in liquid ethane and placing the resulting specimen into a cryo-TEM for imaging. Once recorded, images of individual liposomes in each electron micrograph can be analyzed using image analysis software in order to determine shape characteristics and size distributions. Analyzing many images will yield statistically valid values that can then be used to evaluate the product quality. Specific image analysis procedures are application dependent and are not addressed in this practice.

5. Significance and Use

5.1 Cryo-TEM is a technique used to record high resolution images of samples that are frozen and embedded in a thin layer of vitrified, amorphous ice (**2-5**). Because vitrification occurs so rapidly, the resultant specimen is almost instantly frozen, yielding a very accurate representation of the specimen at the

moment of freezing, without the distortions typically associated with air drying delicate wet samples. Once frozen, images of the specimen are recorded at low temperature using a specially designed electron microscope equipped with a cryo-holder capable of operating under low dose conditions in order to prevent beam induced structural damage to the specimen. The cryo-TEM technique is the consensus choice to directly observe, analyze and accurately measure liposomes suspended in aqueous solutions. Fig. 1 illustrates this by comparing an electron micrograph from an air-dried negatively stained liposomal preparation with an electron micrograph of the same solution imaged by cryo-TEM.

5.1.1 Fig. 1 demonstrates that liposomes may become distorted and are difficult to measure and analyze when they are air-dried, while the same liposomal preparation is clearly easier to analyze when the specimen is near-instantly preserved by vitrification.

5.1.2 Cryo-TEM involves applying a small volume of sample to a specially prepared holey, ultra-thin or continuous carbon grid suspended in a cryo-TEM plunger over a cup of liquid ethane cooled in a container filled with liquid nitrogen (**2**, **3**). These grids can be purchased or prepared in the laboratory using a carbon evaporator with glow discharge capabilities. Once the sample has wet the surface of the grid, and sufficient time allowed for the solution to equilibrate with regard to liposome spreading over the grid surface, the excess is wicked off (blotted) with filter paper and the grid plunged into the liquid ethane, vitrifying the sample. Once frozen, the sample is maintained at a liquid nitrogen temperature while it is imaged in a cryo-TEM operating under low electron dose conditions. There are several limitations associated with implementing this technique to analyze liposomes:

5.1.2.1 *Thick Ice*—The vitrified ice thickness is often determined by the sample or the cryo-TEM procedure itself. Large liposomes, defined to include larger structure and sizes with respect to this practice, are generally associated with thicker ice, while smaller liposomes (structure and sizes) are associated with thinner ice. Generally, thick ice occurs when either excess water forms a thicker ice layer or samples containing larger liposomes are fully covered with water making the ice thicker around the sample. Thicker ice tends to block the electron beam either completely or partially which compromises image quality.

5.1.2.2 *Larger liposomes (structure and sizes) are preferentially lost during sample preparation.* Larger liposomes, defined to include larger structures and sizes with respect to this practice, are more difficult to image for two reasons. The first is the cryo-TEM procedure itself. This procedure requires the use of filter paper to blot away excess aqueous solution from the EM grid just prior to vitrification. The larger liposomes suspended within the sample preferentially wash away from the grid and into the filter paper, ending up in the filter paper. This is perhaps because the larger liposomes have larger surface areas that expose them to relatively larger forces during the rapid flow of the water to the filter paper. This makes them difficult to find and measure in electron micrographs when their relative concentration in the specimen is low, meaning that few are left behind after blotting. The second reason is that larger liposomes that are left behind on the EM grid, are often embedded in thicker ice that is too thick for the electron beam to either penetrate or, if it does, results in images that are too low in quality to provide adequate signal for image processing.

5.1.2.3 *Liposomal Distortion*—Because liposomes are essentially loose membrane bounded fluid compartments, freezing them within a layer of vitrified ice that is thinner than their diameter may cause the surface tension on both sides of the specimen to compress some of the liposomes leading to various levels of flattening distortions. Accurate size measurements of such distorted liposomes would require volumetric measurements of all the liposomes within a field of view through a three-dimensional analysis using electron tomography.

6. Reagents and Equipment

6.1 Purified *preparation of liposomes* suspended in an aqueous solution.

6.2 *Cryo-TEM plunger*, commercial or homemade.

6.3 *Ethane gas*, research or higher purity grade.

6.4 *Pipetter*, set to 3–4 µL.

6.5 *Pipette tips*.

6.6 Four *brass or copper rods*, approximately 5 mm (diameter) × 12 cm.

6.7 *Liquid nitrogen*.

6.8 *Filter paper*, cut into wedges approximately 2 cm in length.

6.9 A suitable number of *200 to 400 mesh holey*, such as lacey, continuous, or ultra-thin carbon-coated TEM copper grids. These grids contain hole patterns etched into the carbon that come in specific hole size, pattern, and frequency. These grids are commercially available from electron microscopy supply companies.

6.10 *Glow discharge device*.

6.11 *Cryo-TEM*, capable of low dose imaging and with an anti-contaminator.

6.12 *Cryo-TEM holder*.

6.13 *Liquid nitrogen grid storage device*.

7. Hazards

7.1 An independent hazard assessment should be conducted by the user of this practice in order to establish the proper safety protocols necessary to implement this standard. The known hazards include, but are not limited to, the following:

7.1.1 *Ethane Gas:*

7.1.1.1 Is an extremely flammable gas.

7.1.1.2 May displace oxygen and cause suffocation.

7.1.1.3 Will be used as a cryogen with a heat capacity of 68.5 J/(mol K) at −179 °C, which may cause frostbite on contact with bare skin.

7.1.1.4 Will be dispensed from a high pressure tank and may be an explosion hazard if heated.

7.1.1.5 May form explosive mixtures with air.

7.1.1.6 Use suitable personal protective equipment (latex gloves, safety glasses with side-shields, and a laboratory coat)

as established by a hazard assessment when working with ethane. Work must be conducted in a ventilated space.

7.1.2 *Liquid Nitrogen:*

7.1.2.1 May displace oxygen and cause suffocation.

7.1.2.2 Is a refrigerant and may cause frostbite.

7.1.2.3 Use suitable personal protective equipment (insulated protective gloves, safety glasses with side- shields, and a laboratory coat) as established by a hazard assessment when working with liquid nitrogen. Work must be conducted in a ventilated space.

7.1.3 *Liposomes:*

7.1.3.1 Some liposomes to be examined by this practice may be toxic.

7.1.3.2 Use suitable personal protective equipment (latex gloves, safety glasses, properly fitted respirator, and a laboratory coat) as established by a hazard assessment when working with liposomes, and handle liposomes in a fume hood when possible.

8. Procedure

8.1 *Grid Selection:*

8.1.1 There are many types of grids that can be used for cryo-TEM. They may be purchased from a manufacturer or can be prepared in the lab. The selection is based on the types of carbon supports over the copper grid surface. The grids should be 200–400 mesh copper TEM grids with either a holey, ultrathin, or continuous carbon support on the grid. Fig. 2 below shows the three different types of carbon films used in cryo-TEM that can be suspended over the holes in a standard copper EM grid.

8.1.1.1 *Continuous Carbon Grids*—This type of grid provides a thick layer of carbon for the liposomes to adhere to. Unlike ultrathin carbon, this carbon is self-supporting and should only be used with 400 mesh copper grids because this mesh is smaller and provides better support for the carbon. The carbon reduces static charge of the specimen and these grids provide better support for sample distribution. Unlike ultrathin carbon, however, they are easier to prepare in the lab or can be purchased from a manufacturer. The main disadvantage is that images contain considerable carbon-associated image noise which may reduce contrast and interfere with measurements due to low signal from liposomes in thicker ice.

8.1.1.2 *Holey Carbon Grids*—This is the most common type of support used for cryo-TEM and should be attempted for initial imaging of liposomal samples. These grids can be purchased or made in the lab with a random and variable pattern of holes in the carbon support; or they can be purchased with a specific sized hole arranged in a precise pattern in the carbon film. Either type may be used for imaging liposomal samples. The advantage of this type of grid is that the specimen is recorded within the holes which eliminates the noise from the carbon in the images. The disadvantage is that the vitreous ice layer will slightly statically charge which can cause slight beam induced specimen drift. This type of charging is minimal and will normally not impact imaging of liposomes. The major disadvantages of holey films are that they require higher concentrations of liposomal sample so that enough liposomes remain behind in the holes prior to vitrification and the sample may be less randomly oriented over holes due to liposome interaction with the surface of the water prior to vitrification.

8.1.1.3 *Ultrathin Carbon Grids*—This type of grid provides a very thin layer of carbon for the sample to adhere to. This carbon layer is too delicate and fragile to be placed unsupported over plain 200–400 mesh copper grids. Instead the thin carbon is placed over previously prepared holey carbon grids. These ultrathin carbon grids may be prepared in the lab or purchased from a manufacturer. If purchased from a manufacturer, they must be used according to the original equipment manufacturer (OEM) instructions as the glow discharge procedure has been shown to damage ultrathin carbon. If made in the lab, they must be prepared with a fresh, ultrathin carbon layer to skip the glow discharging step which damages thin films. This type of grid should be used for cryo-TEM when the concentration of liposomes is low or when the liposomes prefer carbon to empty holes. Other advantages of ultrathin carbon grids include less beam-induced charging over those holes, and better sample distribution across the grid. The disadvantage to using this type of grid is that the carbon grains can add some noise to the recorded images. This noise is less than that produced in thicker carbon films (see 8.1.1.1). The disadvantage of using this type of grid is that ultrathin carbon is extremely delicate, requiring the holey carbon film for support. Glow discharging (see 8.2) this type of grid to make the carbon hydrophilic damages the film causing it to be etched or worn away. Ultrathin carbon grids should be prepared fresh to ensure hydrophilicity.

8.2 Glow discharge holey, or continuous carbon grids using a glow discharge device **(6)**. Ultrathin carbon grids should be prepared fresh or if purchased, used according to manufacturer instructions.

8.2.1 The necessary number of grids for cryo-TEM required to conduct the experiment shall be exposed to the plasma generated by a glow discharge device no more than 24 hours prior to use for the procedure outlined in this practice. Without this step, the grid may remain hydrophobic and the sample will not wet the surface of the grid. Exposure to the plasma will be conducted with the carbon film side of the grid (see Fig. 3(*A*), 8.3) facing the plasma, at a vacuum specified by the glow discharge device manufacturer. Exposure will be conducted for 0.5–2 minutes depending upon the length of time required to ensure that the carbon film-coated TEM grids are hydrophilic.

8.3 *Preparation of the Cryo-TEM Plunger:*

8.3.1 Either a commercially manufactured or homemade cryo-TEM plunger is suitable for this practice.

NOTE 1—The operation of commercial plungers must follow the manufacturer's operating instructions and take into account any hazards identified by the manufacturer or established by a hazard assessment. The instructions outlined in this practice will presume use of a homemade style plunger, similar to the guillotine style shown in Fig. 3(*B*).

8.3.2 Pre-cool the ethane cup by pouring liquid nitrogen into the insulated liquid nitrogen reservoir, making sure the level of the liquid nitrogen remains below the lip of the ethane cup. (**Warning—**Liquid nitrogen is a suffocation and frostbite hazard. Work must be conducted as established by a hazard assessment in a ventilated space and care must be taken to avoid prolonged contact with the refrigerant.)